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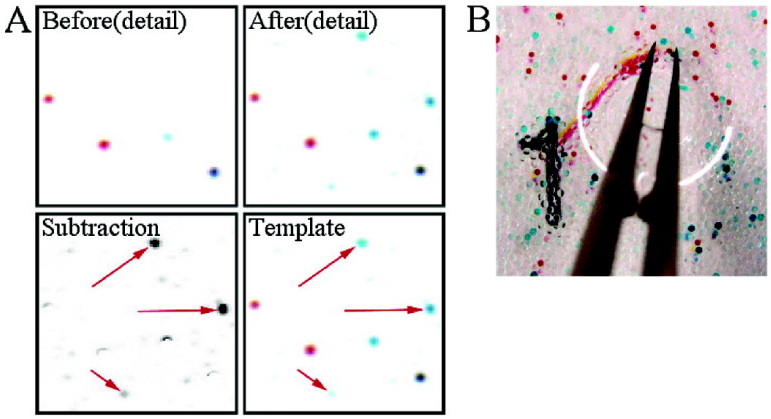

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Image Subtraction Approach to Screening One-Bead-One-Compound Combinatorial Libraries with Complex Protein Mixtures

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To screen one-bead-one-compound (OBOC) combinatorial bead libraries,[1] one generally uses tagged purified protein as the screening probe. Compound beads that interact with the purified protein are then identified, for example, via an enzyme-linked colorimetric assay, and isolated for structure determination. In this report, we demonstrate a rapid and efficient method to screen OBOC combinatorial libraries utilizing two protein mixtures as screening probes, and by comparing optical images of the beads stained by one protein mixture but not the other, ligand beads unique to one of the two protein mixtures can be identified. The significance of this method is that it allows for rapid selection of ligands directed against proteins unique to one mixture while screening out positive beads resulting from proteins common to both mixtures as well as beads that are positive as a result of interactions with chemical and protein components found in the assay itself. The method is fast, efficient, and uses off-the-shelf equipment.

Introduction

We first described the "one-bead-one-compound" (OBOC) combinatorial library method in 1991,[2] in which large compound bead-libraries (millions of compounds) are generated such that each individual bead displays one unique chemical entity. By incubating this OBOC library with a purified tagged-target protein, compound beads that interact with the target protein can be identified and isolated, and the chemical structure of the bead-bound chemical compound can be determined. This combinatorial library method is highly efficient both in library synthesis and in screening. During screening, hundreds of thousands to millions of compounds are exposed not only to the target protein, but also to the other screening reagents, such as secondary antibodies, streptavidin, reporting enzymes, blocking agents, etc. False positive beads could potentially arise due to these secondary reagents or nonspecific binding. One way to overcome such a problem is to physically isolate and rescreen the positive beads with orthogonal secondary reagents so that most of the false positive beads can be eliminated. This approach could be tedious if many false positive beads are present in the initial screen. We subsequently described a dual color screening method in which the beads were screened and labeled with one color in the first step, followed by an orthogonal reagent and a second color substrate in the second step.[3] This approach enables us to eliminate many false positive beads. Buettner et al. subsequently described a similar two-step dual color approach to screen their peptide bead libraries for ligands against Factor IX.[4] In their report, BCIP and NBT were used in the first step and neutral red in the second step. The above screening methods have been used successfully by many investigators to screen purified target proteins against random bead libraries. In principle, these methods can also be applied to screening complex protein mixtures to identify ligands unique to a target protein present within protein extract 1 but not protein extract 2. However, in practice, many false positive beads could emerge from such screening due to many nonspecific interactions between the complex protein mixture and a large number of ligands. Even with the dual color screening methods mentioned above, it is often very difficult to find and isolate the few true positive beads out of thousands of false positive beads. There is a need to develop a highly efficient and simple method that allows the researcher to (i) rapidly identify the few true positive beads from a large number of false positive beads, (ii) identify the compound beads that bind to a predefined binding site on a target protein, (iii) track the color development of every individual bead over time so that the relative baseline color intensity of each bead and relative amount of bound enzyme (alkaline phosphatase) on each bead can be compared, and (iv) compare two complex protein mixtures so that ligands against unique target proteins present in only one of the protein mixtures can be identified. The novel screening method described in this article fulfills all of the above requirements, involves a simple two-stage screening process, and employs an image subtraction technique that enables one to select the desirable compound beads.

The first stage involves screening to colorimetrically mark nonspecific interactions in combinatorial library in the presence of background proteins or protein extract, followed by a second stage of screening and colorimetric marking with the target protein or protein extract. Just prior to the second marking step, the beads are immobilized in agarose and scanned on a flat-bed transparency scanner. After the second marking incubation with appropriate enzyme substrate, the

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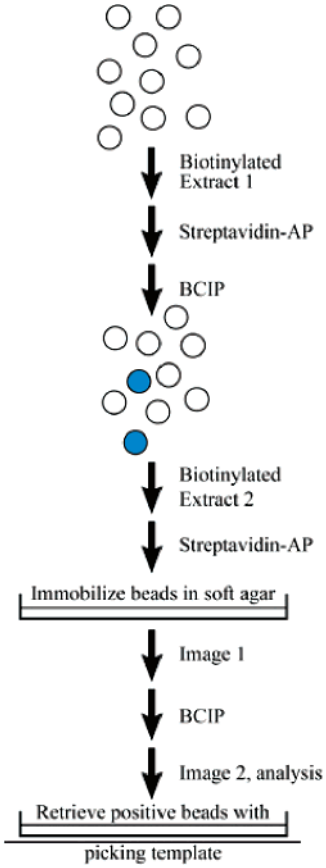


Figure 1. Overall scheme for the two-stage subtraction screening method for one-bead-one-compound combinatorial libraries.

immobilized beads are scanned again. Subtraction analysis of the scanned images generates a template image highlighting the beads unique to the desired target protein. The overall scheme of the screening method is summarized in Figure 1.

Results and Discussion

To verify that the experimental system produces ligands with biological relevance, the image subtraction technique (Figure 1) was first tested by rescreening the same physical OBOC combinatorial peptide library (XXXpXXXX, wherein X = all 19 amino acids and p = D-proline) that had been screened in 1995 with an anti-insulin monoclonal antibody (clone AE906)[5] using the standard enzyme-linked colorimetric screening method.[5] In the standard screening method that was used previously, biotinylated monoclonal anti-insulin antibody was used as the primary reagent, and streptavidin-alkaline phosphatase, as the secondary reagent, followed by BCIP as the colorimetric substrate.[5] Positive beads collected were decolorized, recycled, and restained with streptavidin−alkaline phosphatase alone. All the beads that turned turquoise at this time contained ligands that bind to streptavidin (false positives), and the remaining colorless beads were presumed to be true positives and microsequenced.

In the new screening method, image subtraction analysis was accomplished by first incubating the bead library with the secondary reagent (in this case, strepavidin−alkaline phosphatase conjugate), followed by BCIP. Without removing any colored beads (which could be a very time-consuming and tedious step), the entire bead library was washed (but not decolorized) and incubated with biotinylated

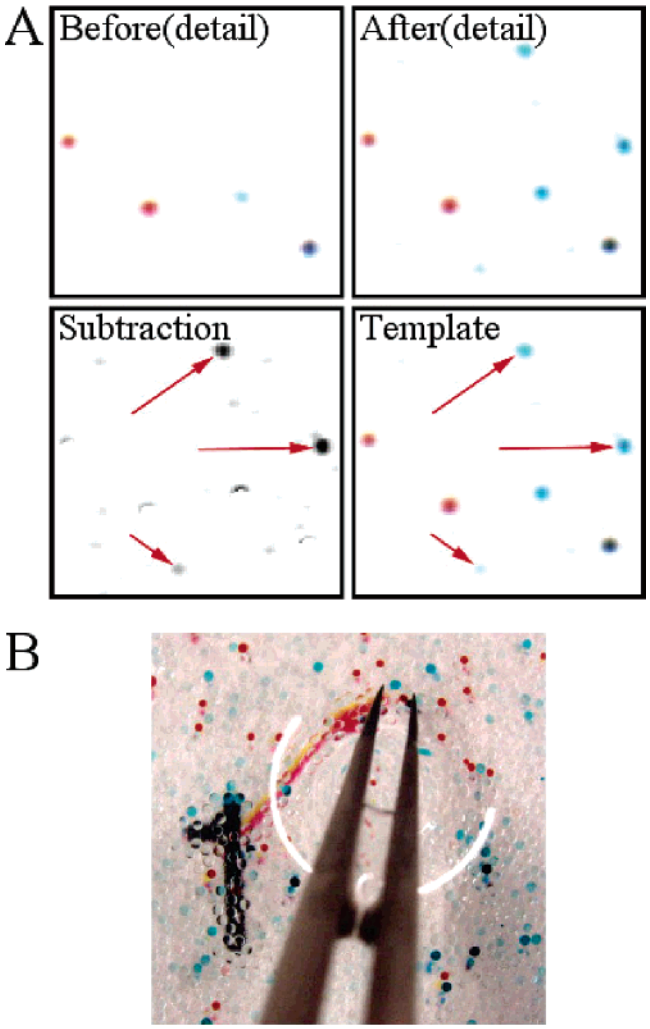
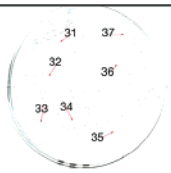
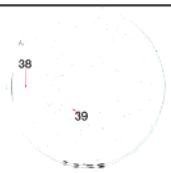


Figure 2. Overview of subtraction analysis. (A) Subtraction images produced on a flat bed scanner. Only a small portion of the entire image is presented. Before and after images correspond to the time points immediately before and 2 h after the addition of BCIP, the second marking step that colors beads unique to the target protein or extract. The subtraction and template images are derived from the before and after images and serve to label beads of interest. (B) The plate with immobilized beads is aligned with the template image. The arrow in the template image points to the bead of interest, which is retrieved with microforceps.

anti-insulin antibody, followed by streptavidin−alkaline phosphatase. The bead library was then immobilized in agarose and scanned (Figure 2A, before). At this stage, only those beads stained in the BCIP reaction were colored (denoted as presubtracted turquoise beads in Tables 1−4). Without moving the plates, BCIP was added for the second time. After 2 h, the plates were scanned again (Figure 2A, after). The before and after scans were then analyzed on a pixel-by-pixel basis using the formula (after − before)/before to generate a subtracted pseudoimage (Figure 2A, subtraction). Arrows were drawn to indicate beads of interest on the subtracted image and were subsequently transposed to the after image to make the template image (Figure 2A, template). The template image was then printed life size and used as a picking template by placing it under a dissecting microscope with the plate containing the immobilized beads placed on top of it and aligned with the template image. To facilitate the alignment of beads between scans, some blank

Table 1. A Summary of the Results and Experimental Conditions Used in the Rescreen of the XXXpXXXX Library with Anti-insulin Ab Utilizing Subtraction Analysis

	Plate A	Plate B	Plate C	Plate D
µg of ST-AP, stage 1	0.25 µg	0.25 µg	0.25 µg	0.25 µg
µg of ST-AP, stage 2	0.1 µg	0.1 µg	0.1 µg	0.1 µg
µg of biotinylated anti-insulin Ab, stage 2	2 µg	0.2 µg	0.04 µg	0 µg
no. presubtracted turquoise beads	2522	2768	2919	3079
no.subtracted positives	>>15	15	8	2
no. ligand sequences obtained	9[a]	7[a]	5[a]	1[a]
Picking templates (Not to scale)				
Ligand sequence	KFQpWGYT INVpWRLS PEQpLWGR KFQpYQRQ KFQpTMHM FTVpGRGD AMTpRRGE TVYpFGGP MFDpFYRK	KFQpYTMD TRQpMFGE VYHpQFTH IQHpVWGG IYMpNWKG LMKpTGYY LTFpHQYY	VLYpYHLT RIHpMDYL PAMpPFGD YNMpNWTL WYTpGKGI	MNMpFTFF

[a] Several beads selected via subtraction analysis did not produce ligand sequence. The advanced age of the XXXpXXXX library resulted in many beads being broken or otherwise irregular. Broken or irregular beads were not submitted for characterization and were responsible for the majority of the missing sequences. Instrument error and human error also accounted for a small fraction of the uncharacterized beads.

Table 2. Summary of the Experimental Results and Conditions from the Screening of Anti-insulin ab against a Linear 6-mer Peptide Library

	plate			
	A	B	C	D
μg of ST-AP, stage 1	0.5	0.5	0.5	0.5
μg of ST-AP, stage 2	0.25	0.25	0.25	0.25
μg of anti-insulin Ab, stage 2	0.2	0.02	0.04	0
no. of presubtracted turquoise beads	934	993	944	847
no. of subtracted positives	7	3	2	1
no. of ligand sequences obtained	7	3	1[a]	1
	Ligand Sequence			
	VTHEMF	SDWNGF	HYQTTY	HSIEFY
	GFNWGH	TFNWAN		
	GFSWTG	YHMDYP		
	AWHDEF			
	WQYIGN			
	VLYNWH			
	VHYPFS			

[a] One bead was deemed irregular and was not characterized.

red Tentagel beads were added to the library as landmarks. The red beads were prepared chemically by derivatizing Tentagel beads with Dabsyl chloride. The arrows on the template now pointed to the beads in the plate that represented subtracted positives. The beads indicated by the arrows in the picking template were retrieved with a pair of hand-held forceps or a micropipet (Figure 2B) and microsequenced. Up to 50 μL of settled 90-μm-diameter TentaGel beads (or ~37 500 compound beads) can be analyzed in one 30-mm Petri dish.

Table 3. The Anti-insulin ab 6-mer Library Screening Experiment Was Repeated in the Presence of Varying Amounts of Insulin

	plate				
	A	B	C	D	E
μg of ST-AP, stage 1	0.3	0.3	0.3	0.3	0.3
μg of ST-AP, stage 2	0.15	0.1	0.15	0.15	0.15
μg of biotinylated anti-insulin Ab, stage 2, MW 150 000	2	2	2	2	2
μg of insulin, MW 2867	10	1	0.1	0.01	0
no. of presubtracted turquoise beads	1799	1913	1607	1857	1959
no. of subtracted positives	1	0	6	6	>7
no. of ligand sequences obtained[a]	1	0	6	1[a]	*a*
Ligand Sequence	IYNQWH		FDWNSG, NWQSGF, IKIHWI, RWEDWM, EFDWNH, YAFDWS	GKGIGF	

[a] Only one bead from plate D was sequenced, and no beads from plate E were sequenced.

Table 4. The XXXpXXXX Library Screened against Anti-Insulin Ab in the Presence of Varied Amounts Mouse Serum[a]

	plate			
	A	B	C	D
μg ST-AP, stage 1	0.3	0.3	0.3	0.3
μg ST-AP, stage 2	0.15	0.15	0.15	0.15
μL mouse serum, stages 1 and 2	20	2	0.2	0
μg biotinylated anti-insulin Ab, stage 2	2	2	2	2
no. presubtracted turquoise beads[b]	4533	5065	1234	809
no. subtracted positives	4	28	>48	2
no. ligand sequences obtained[b]	2	13[b]	*b*	0
Ligand Sequence	GVRpWINM, ANNpGVFR	KEKpALTD, RDGpNKTQ, KKDpPNMY, KIKpEEIG, KIQpGSEE, PARpHVEH, KQNpAWSS, IPKpHGVQ, KHGpEIGQ, KHGpEIGQ, PFApVHNK, ELLpYLRY, HLDpKKNV		

[a] A primary component of mouse serum is IgG, which completes the binding of anti-insulin Ab to the combinatorial library bead. [b] Many beads selected via subtraction analysis did not produce ligand sequence. The advanced age of the XXXpXXXX library resulted in many beads being broken or otherwise irregular. Broken or irregular beads were not submitted for characterization and were responsible for the majority of the missing sequences. Instrument error and human error also accounted for a small fraction of the uncharacterized beads. ND. Not determined. In plate B, only half of the selected beads were sequenced.

The XXXpXXXX library was rescreened utilizing the subtraction analysis method at several anti-insulin Ab concentrations, and the positive beads were retrieved and sequenced. A summary of the experimental conditions is presented in Table 1. Beads with sufficient turquoise color to be detected by the scanner prior to the addition of BCIP were considered "presubtracted turquoise beads" (bind to streptavidin) and those new positive beads identified after 2 h were denoted as "subtracted positives" (bind to anti-insulin Ab). Under the four different screening conditions shown in Table 1, including the negative control without any anti-insulin Ab, the number of "presubtracted turquoise beads" was about the same, ranging between 2500 and 3000. This is expected because these are the peptide beads that interact with streptavidin alone. The number of subtracted positives (a few to a few dozens), on the other hand, increases as the concentration of anti-insulin Ab increases. Some of these positive beads were sequenced. Several motifs were found repeated, with the most common being the N-terminal KFQp that was found in four of 21 sequenced beads. The KFQp motif matches and extends previously published data [5] that showed an FQp motif in three of the published peptide sequences. Of the two beads retrieved from the zero target protein control reaction, one was deemed irregular and not submitted for sequencing, and the other ligand obtained from the zero target protein control experiment did not contain any of the common motifs (Figure 3). The presence of the NMp motif in the zero control sequence (Table 1, plate D) as well as in one sequence from Table 1, plate C, is not unexpected. Randomly obtained sequences (i.e., from the

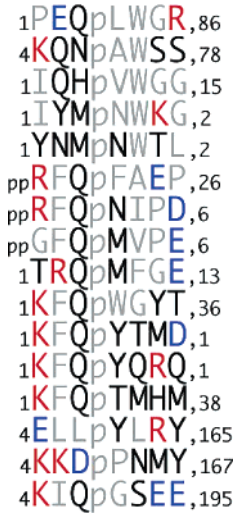


Figure 3. The XXXpXXXX library sequence stack. Amino acid sequences from sequenced combinatorial library beads are arrayed in a stack to present homology patterns. Red denotes positive charges, turquoise denotes negative charges, gray residues are hydrophobic, and black residues are polar. The number at the beginning of the sequence identifies the Table and corresponding experiment from which it was derived (1, Table 1; 4, Table 4; pp, published paper). The number following the sequence denotes the rank in the motif finder output. Lower numbers denote more identity between motifs.

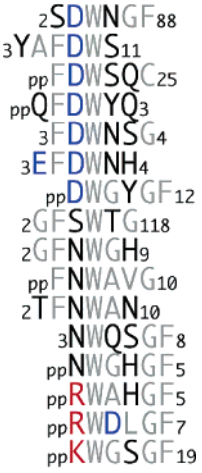


Figure 4. The 6-mer linear peptide library sequence stack. Amino acid sequence from sequenced combinatorial library beads aligned to present homology patterns. In contrast to the previous figure, there are no fixed amino acids in this library, so unaligned motifs are more common. Red denotes positive charges, turquoise denotes negative charges, gray residues are hydrophobic, and black residues are polar. The number at the beginning of the sequence identifies the Table and corresponding experiment from which it was derived. The number following the sequence denotes the rank in the motif finder output. Lower numbers denote more identity between motifs.

zero target protein control) produce consensus matches at reduced frequencies as a function of the number of available sequences to match. Matches are sometimes seen even between randomly generated sets of sequences.

The anti-insulin Ab was subsequently screened with a BBBBBB combinatorial library wherein B represents 19 amino acids (no Cys) and with Arg and Lys present at reduced frequencies. This type of library had also been previously screened. In contrast to the previous experiment, this combinatorial library was newly synthesized. A summary of the experimental conditions and results is presented in Table 2. Despite the use of double the amount of streptavidin-AP in this experiment when compared to the experiment with the XXXpXXXX library (Table 1), the number of presubtracted positives in this experiment was about one-third that of those found in the experiment shown in Table 1. This was not surprising because HPQ and HPM are known motifs for streptavidin,[6] and it was likely that a fixed D-proline in the middle of the random peptide XXXpXXXX library would generate more peptides with turned conformations that resemble that of HPQ (or HPM) than would be generated from a library without either a fixed D- or L-proline. Similar to that in Table 1, the number of subtracted positives in Table 2 increased as a function of increasing concentration of anti-insulin Ab. The amino acid sequence of some of the positive beads also had strong consensus with previously obtained ligands, with FNWA/G being the highest scoring common motif. DW, followed by GF, was also a motif found in both data sets. One bead in the zero target protein control reaction was deemed to be positive. It was retrieved and characterized; however, insufficient sequence similarity with the common motifs was found in this ligand to merit its inclusion in the common motif stack (Figure 4), and the positive appearance of the bead was likely the result of a screening artifact.

We have also performed screening experiments in which varying amounts of insulin were added to block the antigen-binding site of the antibody. Experimental conditions similar to those presented Table 2 (plate A) were used as the base conditions. A summary of results is presented in Table 3. The molar ratio of insulin (MW 2867) to Ab (MW ~150 KD) varies between ~250:1 and 0.25:1. As expected, the number of subtracted positive beads was highest when no insulin was added (plate E). At an insulin/Ab molar ratio of 25:1 (plate B, 1 μg of insulin), no subtracted positives was detected. Six subtracted positives were detected in each of the plates containing 0.1 and 0.01 μg of insulin. Several strong motifs were found in the data set obtained from these two plates. These include NW_GF and FDW, both of which matched previous experiments and previously published motifs.[5] The presence of a single positive bead in plate A was intriguing. The sequence did not contain any of the strong motifs found in previous experiments and could indicate that this ligand was binding to the antibody at a site other than the antigen- or insulin-binding site. In principle, one can incorporate a known competing ligand in stage 1, but not in stage 2, of the screening so that only those ligands that bind to the same pocket on the target protein as the competing ligand will be identified.

The above-mentioned subtraction screening method is particularly useful when the screening probe is a complex protein mixture, such as whole cell extract, rather than a purified protein. For example, one is interested in identifying ligands against a unique protein target present in cell extract 2 but not cell extract 1. By subtracting the bead library image obtained from cell extract 1 from that of cell extract 2, one should be able to identify those compound beads coated with the unique protein present in cell extract 2. Furthermore, one may screen for ligands against a binding site of a specific target protein within the whole cell extract by incorporating

a competing ligand into the cell extract during stage 1 and eliminate it during stage 2 screening. To demonstrate how the subtraction method works with such complex protein mixture, we use mouse serum as "cell extract 1" and the same mouse serum spiked with anti-insulin Ab as "cell extract 2". Our goal is to use the subtraction screening method to identify peptides that bind to the monoclonal anti-insulin Ab but not to the polyclonal immunoglobulins or other proteins present in the serum. Because both the anti-insulin Ab and the mouse serum were biotinylated, the overall number of presubtracted turquoise beads increased dramatically (compare the number of presubtracted turquoise beads in Table 4 vs that in Table 1). Despite the increase in background, we were still able to identify ligands that showed consensus with previously characterized ligands (Figure 3).

A rebinding assay was performed to test the binding of the newly characterized ligands side-by-side with the previously published ligands. Rebinding results indicate that all of the ligands bound to anti-insulin Ab. To rank the strongest Ab binders from the peptide ligands, more stringent rebinding assays with reduced anti-insulin Ab concentrations were performed. Since streptavidin is also a component of the rebinding assay, it was essential that the rebinding assay be done under conditions that do not result in streptavidin's binding directly to the peptide ligands. Results indicate that under stringent conditions, streptavidin failed to bind the peptide ligands in the absence of Ab under conditions that are favorable for anti-insulin Ab binding. The binding affinity of the newly discovered ligands was comparable to that of the previously published ligands when assayed with a stringent rebinding assay (DNS).

To facilitate the analysis of motif patterns found in the characterized ligands, we have designed a software program to find and score common motifs. Because motifs can be discontinuous, the motif-finding program (named *mfer*) only scores exact matches and does not penalize for mismatching amino acids. Likewise, the similarity motif scoring program (named *simfer*) scores for similarity and identity but does not penalize for mismatches. Sequence from both the XXXpXXXX and BBBBBB experiments were scored together to gauge the amount of motif overlap, if any, in the two libraries. The majority of the best matches are found in the BBBBBB library. The highest-scoring ligands in the XXXpXXXX stack (Figure 3), KFQpYTMD and KFQpYQRQ, share the motif KFQpY and have identity at 5 of 8 positions. It is also the top match in the similarity scoring. The previously published ligands RFQpNIPD and GFQpMVPE are the sixth-best match and share the motif FQp_PD/E. The highest scoring match between the previously published data and experiments presented herein is the 13th-best match overall and has identity at four of eight positions.

Similar matching is seen with the BBBBBB library (Figure 4). FDW and N/RW_S/HGF are the two major motifs found in both the current experiment and the previously published data. There is also increased homology between the ligands found in the BBBBBB library when compared to the XXXpXXXX library. This is the expected result. The XXXpXXXX library has a larger number of permutations; therefore, a smaller percentage of the total number of permutations was screened in each experiment that utilized the XXXpXXXX library. This makes it harder to determine consensus in the XXXpXXXX vs the BBBBBB libraries when the same number of beads are screened from each.

As indicated earlier, the XXXpXXXX library was at least 10 years old. Nevertheless, we were still able to obtain meaningful data from such an aged library. However, it is important to point out that the physical structures of the beads in this aged library do deteriorate, as reflected by the presence of a significant number of broken beads in the library.

In the experiments reported here, we only scanned the plates 2 h after the addition of BCIP. Since then, we have also exploited the method by scanning the plates multiple times over the 2-h period. This enables us to study the kinetics of color development of each bead. This is important because it allows us to select those beads with the most bound alkaline phosphatase, that is, those beads that turn darkest in the shortest time.

Conclusions

A rapid and efficient method to screen OBOC combinatorial libraries utilizing two protein mixtures as screening probes has been developed and validated. The method uses common reagents and equipment to rapidly eliminate false positive beads produced by the screening reagents while highlighting combinatorial beads that bind uniquely to the target protein of interest. This method produces results that are similar to previously published work utilizing older methods. Moreover, this method produces ligands with sequences similar to previous work, even in the presence of large amounts of other proteins, of which their ligands can be identified and eliminated through image subtraction. On the basis of our experience, we expect other laboratories may find this method useful. Numerous studies utilizing this method are ongoing in our laboratory.

Experimental Section

General Method. Bead libraries were generated on TentaGel from Rapp Polymere. Amino acids used in bead library synthesis were from Synpep. Antibodies were biotinylated with EZ-Link sulfo-NHS-LC-biotin from Pierce. Bead library screenings were performed in disposable polypropylene columns from Perkin-Elmer Life Sciences. All buffer reagents were from Sigma unless otherwise noted. PBS, TBS, and BCIP buffers were as described in ref 5. Bead staining utilized BCIP from Bio Synth AG, which was hydrolyzed with streptavidin−alkaline phosphatase conjugate from Zymed. Bead immobilization was performed in SeaPlaque agarose from Bio Wittaker Molecular Applications in 52-3001 Petri dishes from Falcon. Scanned images were generated on a Umax Astra 2400S flatbed transparency scanner. Computer imaging was accomplished with Adobe Photoshop and the C programming language. The compiler was GCC 3.3. Amino acid sequencing was performed on an Applied Biosystems Procise 494 Protein Sequencer.

Library Synthesis. One-bead-one-compound XXXpXXXX (turn) and BBBBBB (linear) libraries were synthesized essentially as described[1] using the "split synthesis approach" in which X denotes any L-amino acid except for cystein and p is D-proline. B denotes any amino acid except

cystein and with the ratio of arginine or lysine reduced 10 fold. TentaGel was used as a solid support, and standard Fmoc chemistry was used for the solid-phase peptide synthesis reactions.[7,8]

Antibody Preparation and Biotinylation. HB125 anti-insulin antibody was dialyzed into PBS buffer and biotinylated with NHS-sulfo-LC-LC-biotin essentially as described by the manufacturer (Pierce). Briefly, 200 μg of Ab was incubated with 5 mol equiv of NHS-sulfo-LC-LC-biotin for 30 min at 23 °C. The reaction was dialyzed, and the biotinylation was verified by Western blot using avidin−HRP (Bio-Rad) essentially as described by the manufacturer.

Library Screening. Four experiments were conducted. On average, 25 000 OBOC beads were screened in each experiment. All bead reactions were performed in 1.5-mL polypropylene disposable microcolumns with inside dimensions of 37 mm × 6 mm (Perkin-Elmer Life Sciences). Unless otherwise noted, all bead reactions were incubated on a Labquake benchtop rotator (Barnstead/Thermolyne) for 1 h at room temperature. Bead libraries were stored in PBS with 0.05% sodium azide. All incubation buffers and components were thoroughly mixed before addition to the column containing the bead library.

Bead reactions are initiated by the placing 25 000 beads in a 1.5-mL column and blocking nonspecific protein binding in PBS + 0.1% Tween-20, 0.1% BSA (Fisher) and 0.05% sodium azide (PBSTBNaN_3) for 1 h. Following the preblock, the bead reaction was washed three times by the addition of 1.5 mL of PBSTBNaN_3, followed by vacuum-assisted removal of the wash buffer. The level of the buffer solution in the column was maintained above the level of the beads at all times. At no time were the beads allowed to become dry. After preblocking, the beads were incubated with antibodies as described in the text in 1 mL of PBSTBNaN_3 for 1 h and then washed three times in PBSTBNaN_3 to remove unbound or loosely associated protein. The beads were then incubated for 1 h with 0.3 μg of streptavidin−alkaline phosphatase in 1 mL of PBSTBNaN_3. Following the streptavidin−alkaline phosphatase incubation, the beads were washed two times in TBS and once in BCIP buffer to remove unbound streptavidin−alkaline phosphatase and to replace a phosphate-based buffer with a Tris-based buffer. Beads with bound serum protein were highlighted by staining with 5-bromo-4-chloro-3-indolyl phosphate (BCIP) at a final concentration of 0.165 mg/mL in 1 mL of BCIP buffer. After incubation for 1 h, beads with bound protein are stained varying shades of turquoise through the action of the attached streptavidin−alkaline phosphatase conjugate. The BCIP reaction was terminated by washing three times with PBSTBNaN_3 to remove unreacted BCIP.

Following staining of mouse serum-positive beads, the bead library was incubated with 2 μg of biotinylated anti-insulin Ab in 1 mL of PBSTBNaN_3 for 1 h then washed three times in PBSTBNaN_3 to remove unbound or loosely associated protein. The beads were incubated a second time, as above, for 1 h with 0.15 μg of streptavidin−alkaline phosphatase per reaction and then washed two times in TBS and once in BCIP buffer. After washing, ∼250 red marker beads were added to serve as reference points in subsequent steps. Prior to the second staining step, the beads were immobilized by preparing a solution consisting of 1% low-melt SeaPlaque agarose in 1 mL of BCIP buffer. The agarose solution was cooled to ∼45 °C, and 1 mL was injected through the base of the inverted column and captured in the lid of a Falcon tissue culture dish. The dimensions of the lid were 35 mm width × 5 mm height. The bead−agarose solution was spread evenly across the surface of the lid with a gentle shaking action before being allowed to cool and harden. After the agarose bead solution had hardened, 1 mL of BCIP buffer was added to the lid and the lid and buffer were allowed to sit for 15 min. During this time, the agarose pulled away from the edge of the lid somewhat. After 15 min, the BCIP buffer was removed with gentle suction, and the lid containing the agarose beads was placed on a flatbed scanner set up for transparency scanning. One milliliter of BCIP buffer containing 0.165 mg/mL BCIP was gently added dropwise on top of the agarose in the lid on the scanner, then the lid and beads were immediately scanned at 1200 dpi with no sharpening or color adjustments using the transparency scanning mode. The resultant RGB image was saved and designated image A. After 2 h, the beads were scanned again, and the second scanned image was designated image B. After scanning, the BCIP reaction was stopped by the addition of 1−2 drops of 1 N HCl and gentle rocking of the lid to mix the acid with the BCIP buffer. The addition of 1−2 drops of acid lowers the pH below the optimum for alkaline phosphatase and essentially stops the reaction. Scanned plates were stored covered in a humid environment until needed. When necessary, dH_2O was added dropwise to prevent agarose dehydration.

Image and Data Analysis. Data analysis was accomplished with code written in C (source code available upon request). Proper analysis of images A and B required that the images be of the same size and that all common points in the two images were aligned so that they could be precisely overlaid. Image alignment and manipulation was accomplished with Adobe Photoshop. After alignment, the images were converted to gray scale and saved in Photoshop raw image format. Either the RGB image can be converted directly to a gray scale image or the red channel in a RGB image can be used if increased sensitivity is needed. Analysis performed in this report was accomplished exclusively with data in the red channel. Data analysis of the image pairs was accomplished with the use of custom C code. Briefly, the C program created three arrays. The size of each of the arrays, in bytes, was determined by multiplying the height (in pixels) by the width (in pixels). Images A and B were then loaded into the first two arrays, and the formula $(B - A)/A$ was applied to each pair of corresponding points. Division by zero errors were avoided by adding one where necessary. The numerical results of $(B - A)/A$ were placed in the third image array at the same relative point in the array at which the A and B values resided. Following these calculations, the values in the third array were written out in raw image format.

The contents of the third data file were opened as a raw image file, converted to RGB, and used as a template to choose positive beads unique to the plasma incubation. This

was accomplished by a visual analysis of all points corresponding to a particular bead in the template image as well as images A and B. Beads that were deemed "whole" in the template image were confirmed as unique to image B by side-by-side comparison of images A and B. Arrows pointing toward beads of interest were drawn on a new image layer in the template image. This image layer was then overlaid onto image B, and the combined image was printed and used as a picking guide for retrieval of the beads of interest. To isolate beads of interest, the lid containing the beads immobilized in agarose was placed on top of the picking guide and then both were placed under a dissecting microscope. Gel loading pipet tips or Dumont #5 Student Forceps (Fine Science Tools) were used to retrieve individual beads from the agarose.

Bead counting was performed in a similar manner. The portion of the scanned color image corresponding to the plate to be counted was first isolated, then split into the component RBG color channels with Photoshop, and the red channel (to count the turquoise beads) was saved as a .raw file. Artifacts such as dust particles and the edge of the plate were removed from the image so that what remained was exclusive to the colored beads. Bead counting and measurement of bead color intensities were then generated by loading the image into a 2D array and then computationally comparing the numerical values for each pixel to the values of the eight adjacent pixels in the image. Whenever a value was found that did not have a higher adjacent value, that pixel was deemed to be the center, or most intense, portion of a bead, and its intensity was recorded. After processing the entire image, the results were binned in steps of ten, charted, and compared to data from the other plates. The subtraction and counting code are available by request.

Determination of Primary Amino Acid Sequence. After retrieval, beads of interest were washed sequentially in 8 M guanidine−HCl, 8 M guanidine−HCl, dH_2O, and dH_2O prior to submission for automated peptide sequencing.

Sequence Motif Analysis. Ligand identity and similarity were analyzed with the help of custom-written perl scripts. Briefly, the scripts find and display sequence matches between the amino acid sequences of the ligands obtained from the subtraction analysis. The most commonly occurring motifs are highlighted, even though they may be discontinuous matches. The script works by breaking each ligand down into all possible smaller combinations containing two or more letters. For example, the ligand FRED would be broken down into - -ED, -R-D, -RE-, -RED, F--D, F-E-, F-ED, FR- -, FR-D, FRE-, and FRED. Likewise, for the ligand FORD. The ligand combinations are then mixed and sorted. The sorted output places the combinations F--D and F--D from FRED and FORD next to each other, thereby identifying common motifs. Although a human can easily pick out motifs from FRED and FORD, the script is capable of rapidly finding all common motifs in hundreds of ligands at once. A variant of the script adds a scoring function based on the PAM30 matrix to identify and score similar motifs. Care needs to be taken that the resultant matches represent significant matching above what would be expected to match in a random data set of equivalent size and length. Random data can present deceptively similar matches with unanticipated frequency. In all cases, the data presented here presented matches at a rate significantly higher than expected from random data. The code is available on request.

Rebinding Assay. Ligands were first resynthesized on TentaGel in bulk. Whereas in combinatorial library synthesis, each bead carries a different sequence, in the retesting assay, each bead carries the same peptide sequence. On average, 1500 same-sequence beads were screened under each set of conditions for each ligand. All bead reactions were performed in 1.5-mL polypropylene disposable microcolumns with inside dimensions of 37 mm × 6 mm (Perkin-Elmer Life Sciences). Unless otherwise noted, all bead reactions were incubated on a Lab quake benchtop rotator (Barnstead/Thermolyne) for 1 h at 23 °C. Beads were stored in PBS with 0.05% sodium azide. All incubation buffers and components were thoroughly mixed before addition to the column containing the bead library.

Bead reactions are initiated by placing 1500 beads in a 1.5-mL column and blocking nonspecific protein binding in PBS + 0.1% Tween-20, 0.1% BSA (Fisher), and 0.05% sodium azide (PBSTBNaN_3) for 2 h. During this time, three pools of protein are set up. Pool C is a target protein blank containing only streptavidin−alkaline phosphatase in 100 μL of PBSTBNaN_3. Pools A and B are the same as C, but also include biotinylated anti-insulin Ab as specified. Streptavidin−alkaline phosphatase and anti-insulin Ab and are allowed to preincubate so that streptavidin may bind the biotin on the Ab for 1 h. Following the prebinding, excess biotin was added to the protein pools to bind and occupy the biotin binding site in streptavidin.

Following the bead preblock, the bead reaction was washed three times by the addition of 1.5 mL of PBSTBNaN_3, followed by vacuum-assisted removal of the wash buffer. The level of the buffer solution in the column was maintained above the level of the beads at all times. At no time were the beads allowed to become dry. After preblocking, the protein pools were diluted up to 1 mL per column, and then 1 mL of the appropriate pool was added to the ligand beads in each column. Following a 1 h incubation, the beads were washed two times in TBS and once in BCIP buffer to remove unbound anti-insulin Ab. Beads with bound anti-insulin Ab were highlighted by staining with 5-bromo-4-chloro-3-indolyl phosphate (BCIP) at a final concentration of 0.165 mg/mL in 1 mL of BCIP buffer. After incubation for 1 h, beads with bound anti-insulin Ab are stained varying shades of turquoise through the action of the attached streptavidin−alkaline phosphatase conjugate. The BCIP reaction was terminated by washing three times with PBSTBNaN_3 to remove unreacted BCIP.

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